REVIEW ARTICLE

Protein kinase C isoenzymes: divergence in signal transduction?

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INTRODUCTION

The development and life-time of multicellular eukaryotic organisms represents a complex interplay of numerous proliferation and differentiation events that proceed in a highly ordered manner. As a prerequisite for those events, cells must respond to extracellular *signals* with a specific set of mechanisms that regulate or modulate *gene expression*. Between the signal and the gene, a system of rather different cellular components is assembled to guarantee a specific and successful process of *signal transduction*. Pathways of signal transduction, though differing remarkably in their complexity and in the use of cellular components, seem to obey certain principles which are evolutionarily conserved and ubiquitously distributed amongst living organisms.

Extracellular signals, so-called *ligands*, either penetrate the cellular membrane or bind to the extracellular domain of *receptors*. Activated receptors as such, or in association with so-called *transducers*, are capable of activating *effectors*–either directly or by means of changing the amount or intracellular distribution of so-called *second messengers*. These second messengers activate target proteins which, as such, or by acting on further 'downstream' targets, finally modulate gene expression at both the transcriptional and translational levels. Target proteins at the same time act back on transducers and/or receptors to switch off the signal transduction in a kind of feedback inhibition. Figure 1 schematically summarizes the pathways of signal transduction (for review see Parker, 1991; Karin, 1992). In view of the complex interplay of signal transduction components that regulate proliferation and differentiation events, it is not surprising that most if not all known proto-oncogenes have turned out to represent proteins involved in signal transduction pathways at all levels, i.e. ligands, receptors, transducers and effectors (for a review see Hunter, 1991). Most of the components of signal transduction pathways are proteins whose activity is altered by either ligand or second messenger binding, by covalent modifications and by subsequent changes in conformation or subunit number. The majority of covalent modifications observed are phosphorylations on tyrosine or serine/threonine residues, and both Tyr- or Ser/Thr-specific kinases and their protein substrates are present amongst the numerous components of signal transduction. Moreover, some of these protein kinases are themselves substrates of other protein kinases or modulate their own activity by (multiple) autophosphorylation reactions.

Two prominent Ser/Thr-specific kinases, both activated by second messenger action, play a central role in signal transduction: the cyclic AMP-dependent protein kinase A (PKA) (for a review see Taylor et al., 1990) and the Ca^{2+}/phospholipid-activated protein kinase C (PKC) (this Review). The latter emphasizes its role as a key enzyme in signal transduction by the fact that it represents the direct "receptor" protein of phorbol esters, substances known to interfere dramatically with proliferative and differentiation events by promoting oncogenic transformation of cells *in vivo* and *in situ*. (To avoid misunderstanding we define data from intact animals or tissue as *in*

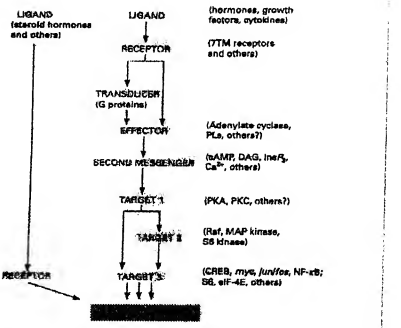

Figure 1 Schematic overview of the signal transduction pathway

Extracellular ligands bind to their cellular receptors. Hydrophobic ligands such as steroid hormones diffuse through membranes and bind to intracellular receptors that directly activate transcription. Activated receptors consisting of a structure with seven transmembrane domains (7TM receptors) interact with a transducer which in turn stimulates an effector. Activated receptors with Tyr kinase activity can directly activate several effector systems. The activated effectors generate second messengers which activate a target cascade leading to gene expression. In many cases PKC is the first target. For a complete description of signal transduction see Parker (1991), Hunter (1991) and Karin (1992).

Abbreviations used: AP, activator protein; CREB, cyclic AMP response element binding protein; DAG, *sn*-1,2-diacylglycerol; DMSO, dimethyl sulphoxide; EGF, epidermal growth factor; eIF, eukaryotic initiation factor of protein synthesis; GAP, GTPase activating protein; IFN, interferon; IL, interleukin; MAP, mitogen-activated protein (kinase); MBP, myelin basic protein; PKA, protein kinase A; PKC, protein kinase C; cPKC, "conventional" PKC; nPKC, "novel" PKC; PL, phospholipase; PMA, phorbol 12-myristate 13-acetate (= TPA); Sp1, stimulatory protein 1; SRE, serum-response element; S6, ribosomal protein 6 of the 40 S ribosomal subunit; TRE, TPA-response element.

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Table 1 PKC isoform cDNAs

All described PKC cDNAs are listed. "+" stands for the complete coding region. The isoform ϵ' is parenthesized because no functional protein could be identified.

PKC	Organism	Source	Region	Reference
α	Bovine	Brain	+	Parker et al., 1986a
	Human	Brain	Partial	Coussens et al., 1986
	Rat	Brain	Partial	Knopf et al., 1986
	Rabbit	Brain	+	Ohno et al., 1987
	Rat	Brain	+	Ono et al., 1988a
	Mouse	Fibroblasts	+	Rose-John et al., 1988
	Mouse	Brain	+	Megidish and Mazurek, 1989
	Human	T cells	+	Finkenzeller et al., 1990
βI	Rat	Brain	+	Ono et al., 1988
	Human	Spleen	+	Kubo et al., 1987a
	Rat	Brain	3'-end	Housey et al., 1987
	Rat	Brain	+	Housey et al., 1988
	Rabbit	Brain	+	Ohno et al., 1987
βII	Bovine	Brain	+	Coussens et al., 1986
	Human	Brain	+	Coussens et al., 1986
	Rat	Brain	+	Knopf et al., 1986
	Rat	Brain	+	Ono et al., 1986
	Human	Spleen	+	Kubo et al., 1987a
	Rabbit	Brain	+	Ohno et al., 1987
	Mouse	Brain	+	Tang and Asbendel, 1990
γ	Bovine	Brain	+	Coussens et al., 1986
	Human	Brain	5'-end	Coussens et al., 1986
	Rat	Brain	+	Knopf et al., 1986; Ono et al., 1988a
	Rabbit	Brain	+	Ohno et al., 1988b
δ	Rat	Brain	+	Ono et al., 1988b
	Mouse	Brain	+	Mizuno et al., 1991
	Mouse	Myeloid cells	+	Mischak et al., 1991a
ϵ	Rabbit	Brain	+	Ohno et al., 1988a
	Rat	Brain	+	Ono et al., 1988b
	Mouse	Brain	+	Schaap et al., 1989
(ϵ')	Rat	Brain	+	Ono et al., 1988b
ζ	Rat	Brain	+	Ono et al., 1989a
η	Mouse	Skin	+	Osada et al., 1990
	Human	Keratinocytes	+	Bacher et al., 1991, 1992
	Rat	Lung	+	Dekker et al., 1992
θ	Mouse	Skin	+	Osada et al., 1992

vivo, data from cell culture as *in situ*, and data obtained from experiments with cell-free components as *in vitro*.) The discovery that PKC represents a large gene family of isoenzymes differing remarkably in their structure and expression in different tissues, in their mode of activation and in substrate specificity may enable us to elucidate the key role of PKC isoenzymes in signal transduction and to link PKC isoenzyme action to the modulation of gene expression necessary for changes in the proliferative and differentiation status of eukaryotic cells.

In this Review, we have compiled current knowledge on the action of PKC isoenzymes with the goal of evaluating the possibility that signal transduction pathways lead to quite divergent responses by usage of the different PKC isoenzymes. Within this scope, we have refrained from citing numerous references that are compiled in detailed reviews on PKC which have appeared at regular intervals in the past (Kikkawa and Nishizuka, 1986; Nishizuka, 1988; Kikkawa et al., 1989; Bell and Burns, 1991; Stabel and Parker, 1991; Azzi et al., 1992; Clemens et al., 1992).

THE PROTEIN STRUCTURE OF PKC ISOENZYMES

Originally, PKC was discovered by Nishizuka and coworkers as a histone protein kinase from rat brain that could be activated by limited proteolysis (Inoue et al., 1977), Ca^{2+} and (phospho)lipids (Takai et al., 1979) or phorbol esters and phospholipids (Castagna et al., 1982). From biochemical studies and purifications (Huang et al., 1986a), it soon became clear that PKC represented a group of at least three isoenzymes or isoforms (α, β, γ), but the major breakthrough emerged from cloning the cDNAs of an ever increasing number of PKC isoforms, mostly from brain cDNA libraries (see references in Table 1). So far, the cDNAs coding for nine different PKC isoenzymes have been cloned from different species and tissues or cell lines (Table 1; following the nomenclature of Nishizuka, 1988). They can be divided into two main groups: the Ca^{2+}-dependent or conventional PKCs (cPKCs) and the Ca^{2+}-independent or novel PKCs (nPKCs) (Ohno et al., 1991). The PKC isoforms α, βII, βIII and γ belong to the Ca^{2+}-dependent group, and the isoforms δ, ϵ, ζ, η and θ to the Ca^{2+}-independent group. The recently identified murine (Osada et al., 1990) and rat (Dekker et al., 1992) PKC η is identical with the human PKC L (Bacher et al., 1991, 1992) and we refer to these PKCs as PKC η (Table 1). The PKC ϵ'-cDNA (Ono et al., 1988b) is very likely to represent a partial ϵ-cDNA clone since no ϵ'-protein could be identified by overexpressing the human ϵ'-cDNA in either mammalian or insect cells (H. Hug et al., unpublished work). For a comparison of the mammalian PKC isoenzymes with those from lower eukaryotes, which would exceed the scope of this Review, the reader should refer to Stabel and Parker (1991).

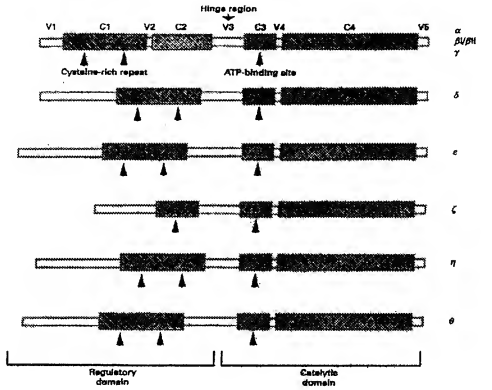

Figure 2 Domain structure of PKC isoenzymes

All PKC isoenzymes consist of constant (C) and variable (V) regions. The cysteine-rich repeats in the C1 region and the ATP binding site in the C3 region are indicated by arrowheads. The arrow points to the hinge region in the V3 domain which separates the regulatory from the catalytic domain.

The primary amino acid structure, deduced from the cDNA sequences available, can be divided into conserved and thus presumably functional domains (C1–C4) which are separated by variable regions (V1–V5), the function of which is not yet evident (Coussens et al., 1986). All PKC isoforms contain these constant and variable regions in a single subunit protein, although the nPKC isoenzymes differ in part from the structure of the cPKCs (Figure 2). The C-terminal regions C3–V5 have been defined in all PKC isoenzymes as the catalytic domain, which is separated by the V3 region from the N-terminal regulatory domain.

The N-terminal V1 region of the cPKC isoenzymes is a short stretch of approximately 20 amino acids and no function has been attributed to this region. In contrast, the V1 region of the nPKC isoenzymes is rather extended and may well influence or modulate the function of the conserved domains common to both c- and nPKC isoforms.

At the beginning of the C1 region, a sequence motif is located that is similar to the consensus sequence xRxxS/TxRx found in the phosphorylation sites of prominent PKC substrates (House et al., 1987; Graff et al., 1989; Kemp and Pearson, 1990; House and Kemp, 1990). However, the serine or threonine residue found in the substrate motif is changed to alanine in all PKC isoenzymes (Figure 3). Thus, this motif cannot be phosphorylated and is very likely to represent a pseudosubstrate site that exhibits autoregulatory features (Soderling, 1990) by blocking the catalytic (substrate binding) site. In fact, pseudosubstrate peptides are rather efficient inhibitors of PKC both *in vitro* and *in situ* (House and Kemp, 1987; Eicholtz et al., 1990; Shen and Buck, 1990); likewise, synthetic peptides containing this sequence with alanine replaced by a serine residue can be used as *in vitro* substrates (Marais and Parker, 1989; Schaap et al., 1989; Olivier and Parker, 1991; Dekker et al., 1992). A PKC α mutant containing a glutamic acid residue at position 25 (Figure 3) showed an increase in effector-independent kinase activity and in its sensitivity towards proteolytic activation (Pears et al., 1990), and anti-pseudosubstrate antibodies could be used to activate PKC *in vitro* (Makowske and Rosen, 1989). On the basis of the pseudosubstrate sequence, one can speculate on the substrate specificity of PKC isoenzymes; however, a comparison of these sites (Figure 3) does not reveal any significant differences and nor do the cPKC isoenzymes α, βI and γ show any distinct differences when probed with the respective substrate peptides (Marais and Parker, 1989). It should be noted, however, that within natural (holoprotein rather than peptide) substrates of PKC, structures of higher order may determine substrate specificity (Kemp and Pearson, 1990).

The Cys-rich region within the C1 domain (Figures 2 and 3) consists of two zinc finger motifs each with six cysteine residues, a DNA-binding motif found in transcription factors like GAL4 (Pan and Coleman, 1990). For both PKC and GAL4, it has been shown that two Zn^{2+} ions are co-ordinated between six cysteine residues (Quest et al., 1992) though no obvious role of Zn^{2+} ions

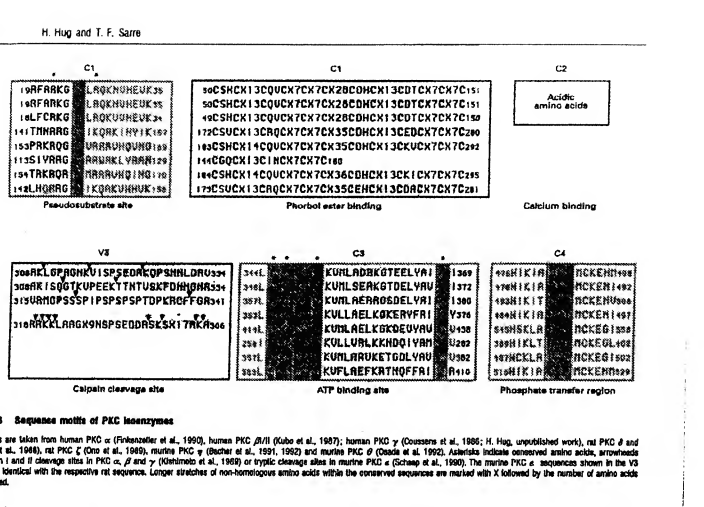

Figure 3 Sequence motifs of PKC isoenzymes

Sequences are taken from human PKC α (Finkenzeller et al., 1990), human PKC βI/II (Kubo et al., 1987); human PKD γ (Goussens et al., 1986; H. Hug, unpublished work), rat PKC β and ε (Ono et al., 1988), rat PKC ζ (Ono et al., 1989), murine PKC η (Bacher et al., 1991, 1992) and murine PKC θ (Osada et al. 1992). Asterisks indicate conserved amino acids, arrowheads the calpain I and II cleavage sites in PKC α, β and γ (Kishimoto et al., 1989) or tryptic cleavage sites in murine PKC ε (Schaap et al., 1990). The murine PKC ε sequences shown in the V3 region are identical with the respective rat sequence. Longer stretches of non-homologous amino acids within the conserved sequences are marked with X followed by the number of amino acids not depicted.

is detectable. For PKC isoenzymes no DNA-binding activity has been demonstrated, but the regulatory subunit alone, generated by proteolytic cleavage in the V3 hinge region (see below), may bind. Three further proteins, diacylglycerol (DAG) kinase (Sakane et al., 1990; Schaap et al., 1990), c-Raf kinase isoenzymes (Bruder et al., 1992) and *n*-chimaerin (Ahmed et al., 1990), show no DNA-binding activity although they contain a zinc finger motif. The use of deletion mutants of different PKC isoenzymes revealed that the Cys-rich region is necessary for DAG and phorbol ester binding (Muramatsu et al., 1989; Kaibuchi et al., 1989; Burns and Bell, 1991). Moreover, the C1 domain, expressed in *Escherichia coli*, exhibited phospholipid-dependent phorbol ester binding (Ono et al., 1989b). PKC ζ contains only one zinc finger (Figure 3) and does not bind DAG or phorbol ester (Ono et al., 1989a; McGlynn et al., 1992); this is in agreement with the finding that, at least *in vitro*, PKC ζ exhibits a constitutive protein kinase activity (Liyanage et al., 1992; McGlynn et al., 1992; Nakanishi and Exton, 1992) which might be influenced *in vivo* by other unknown factors. It seems noteworthy that all PKC isoenzymes, even PKC ζ exhibit a distinct distance of 15 amino acids from the end of the pseudosubstrate box to the beginning of the zinc finger motif (see Figure 3).

The Ca^{2+}-independent nPKCs lack the C2 region (Figure 3), which is thought to represent the Ca^{2+}-binding domain of the cPKCs (Ono et al., 1988b; Ohno et al., 1988a). No sequence motif that represents a known Ca^{2+}-binding site, e.g. the classical E–F hand binding motif, could be identified, but the C2 region contains many acidic amino acids which are thought to participate in Ca^{2+} binding (Ohno et al., 1987). Nevertheless, homologies of the C2 domain of the cPKCs with sequences of other Ca^{2+}-binding proteins have been reported, e.g. with phospholipase A_2 (Clark et al., 1991), phospholipase C-γ (Stahl et al., 1988) and two synaptic vesicle Ca^{2+}-dependent proteins (Perin et al., 1990; Geppert et al., 1991).

The V3 or hinge region separates the regulatory from the catalytic domain (Figure 2). This region is sensitive to proteolytic cleavage by trypsin or the Ca^{2+}-dependent neutral proteases calpain I and II which leads to a constitutively active kinase (Kishimoto et al., 1983; Huang and Huang, 1986; Schaap et al., 1990). PKC α is more resistant to proteolytic digestion than PKC β and γ (Huang et al., 1989; Kishimoto et al., 1989; Kochs et al., 1993). The cleavage sites for PKC α, β and γ by calpain I and II (Kishimoto et al., 1989) and for PKC ε by trypsin (Schaap et al., 1990) have been determined and are depicted in Figure 3, though no obvious consensus sequence can be found between those and other nPKC isoenzymes. Further experimental evidence will be necessary to determine whether or not the V3 region of the different PKC isoenzymes exhibits certain features that modulate the susceptibility to proteolytic cleavage. Recent findings (James and Olson, 1992) indicate that the hinge region (and the catalytic domain) may be involved in the nuclear targeting of PKC α (see below).

The C3 region contains the ATP-binding motif $xGxGx_1Gx_{15}Kx$ conserved in most protein kinases (Taylor et al., 1990; Kemp and Pearson, 1990). Only PKC ζ differs slightly from the consensus ATP-binding motif; it contains an alanine

instead of a glycine at position 264 (Figure 3). Nevertheless, human PKC ζ purified from recombinant baculovirus-infected insect cells does show kinase activity (Liyanage et al., 1992; McGlynn et al., 1992; G. Kochs and H. Hug, unpublished work).

The C4 region contains the substrate binding site and the phosphate transfer region (Figure 3). The central element in the phosphate transfer region, again highly conserved amongst protein kinases (Taylor et al., 1990; Kemp and Pearson 1990), is the sequence DFG. The Asp residue is thought to be responsible for the transfer of the phosphate group to substrates. In all PKC isoenzymes, there is a conserved distance of 105–108 (113 in PKC γ) amino acids between the end of the ATP-binding site and the beginning of the phosphate transfer region. Again, PKC ζ exhibits a slight deviation from the consensus sequence DFG with a substitution of phenylalanine by tyrosine.

THE BIOCHEMICAL PROPERTIES OF PKC ISOENZYMES

Though certain features of the PKC isoenzymes can be deduced from the protein structure derived from the sequence of the cloned PKC cDNAs (see above), the greater body of information has been accumulated by detailed biochemical analyses. These studies were carried out with isoenzymes purified to homogeneity mostly from rat, rabbit or bovine brain (Huang et al., 1986a; Jaken and Kiley, 1987; Sekiguchi et al., 1988; Marais and Parker, 1989; Leibersperger et al., 1990; Koide et al., 1992; Ogita et al., 1992; Saido et al., 1992). To date, expression of isoenzyme cDNAs in either COS cells or recombinant baculovirus-infected insect cells allows for the purification of distinct PKC isoenzymes for biochemical characterization (Knopf et al., 1986; Ohno et al., 1988a; Ono et al., 1989a; Patel and Stabel, 1989; Burns et al., 1990; Fiebich et al., 1990; Schaap and Parker, 1990; Akita et al., 1990; Osada et al., 1990; Olivier and Parker, 1991; Burns and Bell, 1991; Stabel et al., 1991; Liyanage et al., 1992; McGlynn et al., 1992; Osada et al., 1992; Dekker et al., 1992). The molecular mass values of these PKC isoenzymes, either calculated from the open reading frame of the respective cDNA sequence or reported from SDS/PAGE analyses, are listed in Table 2. The obvious deviations of these values are thought to be due to co- or post-translational modifications (see below); in several cases, a doublet of protein bands is observed.

Efficient *in vitro* assays have been developed to study partially or highly purified fractions of PKC isoenzymes derived from the sources mentioned above. As a major advance, the use of DAG/PtdSer vesicles (generated by sonication) as the classical activator of PKC (Takai et al., 1979; Castagna et al., 1982) has been substituted by so-called *mixed micelles* as the activating principle of PKC (Hannun et al., 1985). The latter, composed of (phospho)lipid activator(s) embedded in Triton X-100 micelles, have proven to virtually mimic the situation of the cellular membrane environment of PKC and has minimized artifacts such as damage to vesicle structure by the presence of Ca^{2+} (Bell and Burns, 1991). Originally, histones (HI or III-S), myelin basic protein (MBP) or protamine were used as substrates for PKC isoenzymes (Jakes et al., 1988; Burns et al., 1990), but synthetic peptides derived from the Ala → Ser mutated pseudosubstrate sequence (Marais and Parker, 1989; Schaap et al., 1989; Olivier and Parker, 1991; Dekker et al., 1992) or from known PKC substrates, e.g. the EGF receptor or MBP (House et al., 1987; Yasuda et al., 1990) have proven to be adequate and even more specific substrates. This has been supported by the recent finding that some of the nPKCs, PKC δ, ε and η, display little or no kinase activity on histone, MBP or protamine (Liyanage et al., 1992; Dekker et al., 1992).

By means of those *in vitro* systems, the biochemical properties of the different PKC isoenzymes have been investigated with respect to the activation, autophosphorylation, proteolytic activation/degradation and, last but not least, substrate specificity.

Activation

The finding that PKC represents a (phospho)lipid dependent protein kinase has drawn attention to the cellular glycerolipids, sphingolipids and their metabolic breakdown products (Berridge 1987, 1989) and the enzymes involved in lipid metabolism, i.e. phospholipases and PtdIns kinase(s) (Meldrum et al., 1991). At least for the cPKCs, a model of activation was sufficient and convincing that included (i) the generation of DAG and $Ins(1,4,5)P_3$ from plasma membrane-associated $PtdIns(4,5)P_2$ by the action of phospholipase C, (ii) the release of Ca^{2+} from intracellular storage sites stimulated by $Ins(1,4,5)P_3$, (iii) the binding of Ca^{2+} to the C2 region of PKC and subsequent translocation of the enzyme to the plasma membrane, (iv) where it is activated, via its C1 region, by DAG and PtdSer, the latter being constitutively present in the membrane (reviewed by Bell and Burns, 1991; Gschwendt et al., 1991; Zidovetzki and Lester, 1992). In this model, phorbol ester would mimic the action of DAG and, by its persistance in the cellular membrane, lead to a long-term activation of PKC (Gschwendt et al., 1991; and references cited therein).

Activation of the cPKCs is thought to require DAG as activator and PtdSer as cofactor of activation, the presence of both reducing the Ca^{2+} requirement of PKC to the micromolar range (Lee and Bell, 1991, and references cited therein). For activation with phorbol esters in the case of cPKCs, the presence of Ca^{2+} is not required but lowers the concentration of phorbol ester necessary to obtain full PKC activity (Ryves et al., 1991). As outlined above, members of the nPKC group do not require Ca^{2+} for activation, but require either DAG/PtdSer or PMA/PtdSer, except for PKC ζ which exhibits a low but constitutive activator-independent kinase activity (Liyanage et al., 1992; McGlynn et al., 1992). However, Nakanishi and Exton (1992) have reported a marked stimulation of PKC ζ activity (purified from bovine kidney) by PtdSer or unsaturated fatty acids, e.g. arachidonic acid.

Recently it has been shown that other components of glycerolipid metabolism can be activators of PKC, at least *in vitro* (Lee and Bell, 1991; Chauhan et al., 1991; Orr and Newton, 1992; Kochs et al., 1993). Cardiolipin is able to activate PKC α, βI (Kochs et al., 1993) and ε (Saido et al., 1992). Arachidonic acid (Klikkawa et al., 1989; Burns et al., 1990; Ogita et al., 1992) and even better lipoxin A (Shearman et al., 1989), another lipoxygenase metabolite, are capable of activating PKC βII, γ (Shearman et al., 1989) and ε (Ogita et al., 1992), even in a Ca^{2+}-independent fashion. PKC α can be activated by arachidonic acid only in the presence of Ca^{2+}, whilst PKC βI and δ do not respond at all (Shearman et al., 1989; Burns et al., 1990). Surprisingly, $PtdIns(4,5)P_2$ can substitute for DAG (Chauhan et al., 1991) as an activator of PKC α, βI and γ (Lee and Bell 1991; Kochs et al., 1993), but not of βII (Lee and Bell, 1992), and PtdIns can replace PtdSer as cofactor of activation, at least for PKC α and βI (Kochs et al., 1993). These findings, together with observations that DAG can be generated in an inositol lipid-independent way, e.g. from phosphatidylcholine (Huang and Cabot, 1990; Cataldi et al., 1990) may indicate that some if not all PKC isoenzymes may be activated by different second

Table 2 Molecular mass values of PKC isoenzymes

Calculated molecular masses are from the cDNA sequence; apparent molecular masses are estimated from SDS/PAGE.

PKC	Molecular mass (kDa)		Reference
	Calculated	Apparent	
α	76.8	80–81	Parker et al., 1986; Marais and Parker, 1989; Burns et al., 1990; Borner et al., 1992
βI	76.8	79–80	Ono et al., 1987, Marais and Parker 1989
βII	78.9	80	Coussens et al., 1986, Ono et al., 1987, Burns et al., 1990
γ	78.4	82+84, 79–80, 77+80	Coussens et al., 1986; Patel and Stabel, 1989, Marais and Parker, 1989, Burns et al., 1990
δ	77.5	78, 76+78, 74+76, 77–79	Ono et al., 1988b; McGlynn et al., 1992; Olivier and Parker, 1991; Ogita et al., 1992; Borner et al., 1992; Liyanage et al., 1992
ϵ	83.5	89, 90, 90–91, 93+96, 96	Ohno et al., 1988a; Borner et al., 1992; Schaap and Parker, 1991; Liyanage et al., 1992; Koide et al., 1992; Saido et al., 1992
ζ	67.7	78, 78, 78–80	Ono et al., 1989a; Nakanishi and Exton, 1992; McGlynn et al., 1992; Liyanage et al., 1992
η	77.9	82, 84, 86	Osada et al., 1992; Dekker et al., 1992; Greif et al., 1992
θ	81.6	79	Osada et al., 1992

messengers in a distinct way. Cytokines such as IFN-α, IL-1 and IL-3 have been reported to induce phosphatidylcholine hydrolysis, but not inositol phospholipid turnover (Duronio et al., 1989; Cataldi et al., 1990; Pfeffer et al., 1990), and recently, selective activation of PKC β and ε by IFN-α treatment of HeLa and Daudi cells, respectively, has been demonstrated (Pfeffer et al., 1990, 1991). Thus, the signal-induced production of a distinct second messenger or activator may actually decide which PKC isoenzyme becomes activated or not. Moreover, a detailed study on the activation potency of different phorbol ester derivatives on PKC isoenzymes reveals quite distinct differences, e.g. for PKC δ and PKC βI (Ryves et al., 1991).

Autophosphorylation

Most protein kinases described so far exhibit a rather pronounced autophosphorylation which is often but not necessarily linked to a modulation of kinase activity (Miller and Kennedy, 1986; Galabru and Hovanessian, 1987). Autophosphorylation has also been reported for all PKC isoenzymes known, e.g. PKC α (Pears et al., 1992), βI (Kochs et al., 1993), βII (Flint et al., 1990), γ (Patel and Stabel, 1989; Flebbich et al., 1990), δ (Ogita et al., 1992), ϵ (Koide et al., 1992; Saido et al., 1992), ζ (R. Hummel and G. Kochs, personal communication), η (Osada et al., 1990; Dekker et al., 1992) and θ (Osada et al., 1992). Detailed studies on the role of autophosphorylation have mostly been carried out with cPKC isoenzymes and have revealed that it is an intramolecular reaction at serine and threonine residues on both the regulatory and catalytic domains (Huang et al., 1986b; Mochly-Rosen and Koshland, 1987; Newton and Koshland, 1987). As expected, autophosphorylation has a K_m value for ATP about 10-fold lower than that for substrate phosphorylation and is strictly dependent on the presence of activators (Huang et al., 1986b; Newton and Koshland, 1989). Though it has been reported to affect PMA and Ca^{2+} binding (Huang et al., 1986b) and the K_m value for histone H1 as substrate (Mochly-Rosen and Koshland, 1987), autophosphorylation clearly seems not to be a prerequisite for PKC activity, but rather a concomitant event. A detailed study on PKC βII (Flint et al., 1990) nevertheless revealed certain interesting features of autophosphorylation: (i) all phosphorylated residues were found in variable regions of PKC (see Figures 2 and 3), i.e. V1 (Ser-16, Thr-17) right before the C1 box, V3 (Thr-314, Thr-324) and V5 (Thr-634, Thr-641); (ii) the surrounding amino acid context did not exhibit any consensus sequence nor any similarity to the pseudosubstrate/substrate consensus motif (see above), though some selectivity must exist since threonine and serine residues next to autophosphorylated ones (e.g. Thr-314 and Thr-324) are not phosphorylated (Figure 3); (iii) the distribution of autophosphorylation sites over the N-terminus, the hinge region and the C-terminus indicates a close proximity of these regions to the catalytic centre of PKC, in spite of the fact that the current model of activator binding (a prerequisite of autophosphorylation) postulates a complete or partial unfolding of the PKC molecule (Bell and Burns, 1991; Zidovetzki and Lester, 1992). Whilst PKC βII phosphorylates on both serine and threonine residues (Flint et al., 1990), a comparative phospho-amino acid analysis of highly purified PKC α, βI and ζ revealed almost exclusive autophosphorylation of PKC α and ζ on serine; in contrast, PKC βI autophosphorylates primarily on threonine residues (S. Gaubatz and T. Sarre, unpublished work).

Reports on a potential role of autophosphorylation for the proteolytic activation and degradation of PKC (see below) have been controversial: whilst Ohno et al. (1990) found a severe impairment of PMA-induced downregulation for a kinase-negative mutant of PKC α expressed in COS cells or rat fibroblasts, kinase-negative mutants of PKC α and γ expressed in COS cells could be downregulated by PMA (Pears and Parker, 1991; Freisewinkel et al., 1991). Moreover, inhibition of endogenous PKC activity by the potent inhibitor K252a did not prevent downregulation in Swiss 3T3 cells (Lindner et al., 1991). Nevertheless, autophosphorylation, as one of the immediate early events of PKC activity, may be used as a potential measure of activation (Mitchell et al., 1989; Molina and Asheadel, 1991; Pfeffer et al., 1991; S. Gaubatz and T. Sarre, unpublished work) and may reveal isoenzyme-specific activation in distinct signalling pathways.

Several reports suggest a post- or even co-translational phosphorylation of PKC prior to activation (Borner et al., 1989; Pears et al., 1992). Without this initial phosphorylation, PKC is

inactive and cannot be activated. This observation is supported by the finding that PKC expressed in *E. coli* is inactive (Dietrich et al., 1989) due to the lack of an as yet unidentified PKC kinase present only in eukaryotic cells. In this respect, protein phosphatases become relevant (Parker et al., 1986b), and it is notable that, at least with PKC α, potato acid phosphatase is capable of dephosphorylating PKC without loss of PKC activity, whilst protein phosphatases 1 and 2A completely abolish PKC activity (Pears et al., 1992). If autophosphorylation plays a role at all (if not for activity or proteolytic degradation then perhaps for translocation or substrate specificity), the localization of its target residues within the variable regions might indicate a role that is quite diverse between PKC isoenzymes.

Proteolytic activation and degradation

As mentioned above, PKC was first discovered as a protease-activated protein kinase (Inoue et al., 1977), and only later did it become evident that proteolytic cleavage actually followed activation (Kishimoto et al., 1983). The respective proteases *in vivo* are thought to be Ca^{2+}-dependent neutral proteases I and II (calpains) which are active in the micromolar and millimolar concentration range of Ca^{2+} respectively (Inoue et al., 1977; Kishimoto et al., 1983). Calpains cleave PKC in the V3 hinge region (Figure 3) and thus produce two distinct fragments, a protein comprising the regulatory domain and a protein containing the kinase domain which is catalytically active in the absence of any activators (Kishimoto et al., 1989; Saido et al., 1992). *In vitro*, this proteolytic activation can be achieved by a limited trypsin treatment (Huang et al., 1989; Newton and Koshland, 1989; Schaap et al., 1990; Koche et al., 1993), though *in vivo*, activation of PKC and translocation to the cell membrane is thought to be a prerequisite for proteolytic cleavage. However, it is not yet clear whether further proteolytic degradation serves as a means of preventing continuing kinase activity or if the catalytic fragment, released from the membrane to the cytosol (and possibly to other cellular compartments), is capable of acting as a constitutive, activator-independent kinase. The latter possibility has found support in the recent observation that a PKC α mutant, devoid of the regulatory domain and expressed in COS cells, was selectively translocated to the nuclear envelope (James and Olson 1992). Moreover, one could speculate that the regulatory fragment may now bind – by its zinc finger domains – to DNA (Murray et al., 1987, and references cited therein).

In many cell types, prolonged treatment with phorbol esters results in (almost) complete depletion of cellular PKC (so-called downregulation) which is in favour of the first alternative, i.e. prevention of permanent kinase activity. In other cases, cellular responses involving PKC could be blocked by the protease inhibitor leupeptin, thus indicating a distinct role of proteolytic cleavage (Pontremoli et al., 1990, and references cited therein). With respect to downregulation, PKC isoenzymes exhibit quite extreme differences *in vivo* (see below) which is in agreement with observations *in vitro*. Compared to PKC β and γ, PKC α is relatively resistant to both calpain- and trypsin-mediated proteolysis (Kishimoto et al., 1989; Huang et al., 1989; Kochs et al., 1993). Moreover, tryptic activation of PKC ε not only rendered the enzyme lipid- and PMA-independent but increased its kinase activity towards histone about 10-fold (Schaap et al., 1990). Further work will be necessary to determine the role of proteolytic cleavage with respect to activation, degradation and relocalization to cellular compartments, and substrate specificity of PKC isoenzymes. It should be noted that recent reports indicate a specific role of the degradation of distinct PKC isoenzymes during the process of cellular differentiation. A decrease of PKC ε seems to accompany (or promote?) the differentiation of mouse erythroleukaemia cells (Melloni et al., 1989; Powell et al., 1992), and the differentiation of a neuroblastoma cell line is promoted by an inactivation of PKC α and ε (Leli et al., 1992).

Substrate specificity

For quite a while, our insights into the substrate specificity of PKC have been constrained by the observation that at least the cPKC isoenzymes appeared to be non-specific Ser/Thr kinases *in vitro*. Thus, histones H1 or IIIS, MBP, protamine or any other basic protein or peptide could be used as efficient substrate as long as it contained the phosphorylation site motif xRxxS/TxRx (see above). This contrasts remarkably to several, but not all, Ser/Thr kinases and to the Tyr kinases known so far (for a review see Kemp and Pearson, 1990). Nevertheless, a comparison of PKC isoenzymes and their activities towards distinct substrates *in vitro* has revealed differences that may indicate an even more pronounced specificity towards natural substrates under physiological conditions. Kinase activities of PKC α, βI, βII and γ towards histone IIIS, protamine or MBP are very similar (Marais and Parker, 1989; Burns et al., 1990), though PKC γ has a 2–3-fold lower activity towards protamine or the pseudosubstrate site-derived synthetic peptide of PKC α or β (Marais and Parker, 1989). Members of the nPKC group differ significantly from cPKC isoenzymes in that they exhibit a rather poor kinase activity ($\delta > \eta > \zeta$) towards histone IIIS, MBP, protamine or protamine sulphate (Ono et al., 1989a; Schaap and Parker, 1990; Olivier and Parker, 1991; Saido et al., 1992; Liyanage et al., 1992; McGlynn et al., 1992; Dekker et al., 1992). Thus, it was sometimes necessary to use synthetic peptides derived from the respective pseudosubstrate sequence, the EGF receptor or MBP, to detect kinase activity of nPKC isoenzymes at all (Schaap et al., 1989; Saido et al., 1992; Ogita et al., 1992). This may well explain the difficulties in detecting new PKC isoenzymes by biochemical analysis, if the appropriate substrates are not available.

Several observations indicate that substrate specificity may be more complex than expected. For PKC ε, it could be demonstrated that proteolytic activation (see above) significantly increased its kinase activity towards histone (Schaap et al., 1990) indicating a distinct influence of the regulatory domain. Indeed, genetically engineered fusion of the PKC ε regulatory domain to the PKC γ catalytic domain imposed PKC ε substrate specificity onto the chimaeric enzyme (Pears et al., 1991). A similar observation has been made with PKC ζ: chymotrypsin-mediated proteolysis results in a catalytic fragment which – in contrast to the intact isoenzyme – now accepts the EGF receptor peptide as substrate (R. Hummel and T. Sarre, unpublished work). In some cases, the activator requirement seems to depend on the substrate used; for phosphorylation of protamine or protamine sulphate, neither cPKC nor nPKC isoenzymes require any activator (Bazzi and Nelsestuen, 1987; Liyanage et al., 1992). In order to phosphorylate an MBP-derived substrate peptide, but not the ε-specific pseudosubstrate site-derived peptide, PKC ε seems to require the presence of Ca^{2+} (Saido et al., 1992).

In situ studies by [^{32}P]orthophosphate labelling of cells treated with PMA or other stimuli of PKC-involving signalling pathways have revealed such a large number of putative physiological substrates (for a review see Kikkawa and Nishizuka, 1986) that it is rather difficult to assess their significance in PKC-mediated cellular events. They can be arbitrarily divided into three major classes: (i) proteins involved in signal transduction and PKC activation (e.g. the EGF, T cell and insulin receptors, Ras and

GAP), (ii) proteins involved in metabolic pathways (channels, pumps), and last but not least (iii) proteins involved in regulatory functions concerning gene expression (transcription factors, translation factors). The phosphorylation of various transcription factors by PKC and other signal-transducing kinases has been reviewed recently (Meek and Street, 1992). It should be noted that amongst PKC substrates there might be several other protein kinases like S6 kinase or Raf kinase (see below).

Three prominent substrates, most likely involved in the control of cell proliferation mediated by PKC, should be mentioned, although little is known of the specificity of PKC isoenzymes towards them. The myristoylated, alanine-rich C kinase substrate (MARCKS) is phosphorylated by PKC under several conditions such as macrophage activation or growth-factor-dependent mitogenesis; its phosphorylation leads to a redistribution from the actin filaments of the membrane to the cytoplasm (Hartwig et al., 1992, and references cited therein). Two further PKC substrates, DNA topoisomerase I (Pommier et al., 1990) and lamin B (Hornbeck et al., 1988; Fields et al., 1988), are nuclear proteins and thought to be involved in the control of DNA synthesis; they gain interest in discussing a translocation of activated PKC isoenzymes to the cell nucleus (see below).

EXPRESSION OF PKC ISOENZYMES

The tissue distribution of the PKC isoenzymes has been determined mostly by Northern blot analyses and, more recently, by Western blotting using isoenzyme-specific antibodies. PKC α βI/II, δ, ε and ζ seem to be ubiquitously distributed, e.g. in brain, lung, spleen, thymus and skin (Nishizuka, 1988; Wada et al., 1989; Yoshida et al., 1989; Schaap et al., 1989; Ohno et al., 1991; Wetsel et al., 1992), whilst PKC γ is exclusively found in the central nervous system, e.g. brain (Nishizuka, 1988; Ohno et al., 1991; Wetsel et al., 1992), and PKC η is strongly expressed in skin and lung and only slightly in brain and spleen (Osada et al., 1990; Bacher et al., 1991). PKC θ is predominantly expressed in skeletal muscle and, to a clearly lower extent, in lung, spleen, skin and brain (Osada et al., 1992). Two remarkable deviations from the distribution of the ubiquitous isoenzymes should be mentioned: PKC α and ε seem not to be present in liver (Schaap et al., 1989; Rogue et al., 1990), where PKC β is the major isoenzyme (Rogue et al., 1990); in contrast, no PKC β could be detected in kidney, i.e. renal mesangial cells, though PKC α, δ, ε and ζ are present (Huwiler et al., 1992; see Table 3). Though it is still an open question why all PKC isoenzymes known so far are expressed in brain tissue, the remarkable differences in number and amount of PKC isoenzymes in other tissues make a concept of mere redundancy with respect to signal transduction rather unlikely. On the other hand, one or the other member of the ubiquitously distributed isoenzymes, e.g. PKC α or βI/II, may substitute for each other in different tissues with respect to identical or similar function. There may be even a distinction between isoenzymes for 'house-keeping' functions and those for distinct function in a differentiated, specialized cell, e.g. for PKC θ in skeletal muscle (Osada et al., 1992).

In Table 3, PKC isoenzyme expression patterns of (selected) cell lines from various origins have been accumulated, mostly determined by Northern or Western blot techniques. In accordance with the tissue distribution described above, PKC α, βI/II, δ, ε and ζ seem to be the most ubiquitous isoenzymes, whilst PKC γ is restricted to a neuronal cell line (PC12). Information on PKC η and θ is as yet too limited. Though both PKC α and β are present in most cell lines investigated, in none of them are both missing. Amongst cells of the haematopoietic system, however, PKC α is absent from myeloid cells of different origin (Mischak et al., 1991b), megakaryocytes (HEL) and macrophages (Grabarek et al., 1992; Duyster et al., 1992), whilst PKC βI and βII are absent from mouse and rat fibroblasts, renal mesangial cells, murine erythroleukaemia and neuroblastoma cells. Moreover, the absence of PKC ε appears to be a feature of cells from the myeloid but not erythroid lineage of the haematopoietic system, i.e. megakaryocytes, macrophages, platelets and the promyelocytic leukaemia cell line HL 60. To date, these observations may represent just state of the art or may indicate an exciting script for distinct PKC isoenzymes within the complex network of proliferation and differentiation events of the haematopoietic system (Mischak et al., 1991b). Since a true picture of PKC isoenzyme expression in different tissues and cell lines is rapidly emerging, it can be anticipated that the role of a given PKC isoenzyme in a distinct signalling pathway may become evident once it is possible to define a given cell type unequivocally by its PKC isoenzyme expression pattern and by its signal response potential.

Little is known about how the expression of PKC isoenzymes is regulated, due to the fact that information on the gene and promoter structure is (yet) limited. So far, the promoters of the rat PKC γ (Chen et al., 1990) and the human PKC β (Niino et al., 1992; Obeid et al., 1992) gene have been cloned. The PKC γ promoter contains one binding site for the general transcription factor Sp1 (stimulatory protein 1) but lacks a TATA and CAAT box and therefore is similar to a housekeeping gene promoter. In addition, however, it contains several regulatory elements (reviewed by Locker and Buzard, 1990): two AP2 sites, one AP1 site, one cyclic AMP response element, one enhancer core element and one *c-myc* PRF site. Since AP1 and AP2 sites confer inducibility by phorbol esters, PKC γ transcription may be under positive control by itself or other PKC isoenzymes. The biological function could be that PKC mRNAs are immediately replenished if PKC is activated and subsequently degraded, e.g. during downregulation by PMA.

The PKC β promoter contains no TATA box, but a CAAT box, two Sp1-binding sites, one octamer binding motif site, one AP1 and one AP2 site are found (Niino et al., 1992, Obeid et al., 1992). By deletion analysis, three positive and two negative regulatory regions could be identified in the 1.9 kbp region upstream from the transcription start site of the PKC β promoter. Transcription under the control of these regions seems to be cell type specific (Niino et al., 1992). Comparison with further, not yet isolated, PKC promoters and a detailed analysis by reverse genetics should identify specific elements which could be responsible for the different expression patterns observed, especially for the nerve tissue-specific expression of PKC γ.

The PKC βI and βII isoforms differ in their 3-ends and originate by differential splicing. Genomic clones of the 3-end of the PKC β gene have been isolated (Ono et al., 1987; Kubo et al., 1987b; Couscens et al., 1987). According to the nomenclature of Kubo et al. (1987b) the βI-specific exon lies behind the βII-specific exon and is separated by an intron of 4–5 kbp (Kubo et al., 1987b; Couscens et al., 1987). If PKC β is expressed at all in a given tissue or cell line (Table 3), one splice form seems to be preferred, which indicates that the main regulation seems to occur at the level of alternative splicing. Differential splicing is also proposed for the PKC ε locus (Schaap et al., 1990); however, only one functional protein derived from the ε locus has been identified so far.

Soon after the detection of the various PKC isoenzymes it became clear not only that there are tissue-specific patterns of expression but that the amount and number of PKC isoenzymes varied within a given tissue depending on its developmental stage

Table 3 Expression of PKC isoenzymes in different cell lines

Cell lines: NIH 3T3, mouse fibroblast cell line; renal mesangial cells, rat renal mesangial cell line; HeLa, human carcinoma cell line; preB cells, B cells, plasmacytoma, myeloid cells, see Mischak et al. (1991b); T cells, human peripheral blood-derived T lymphocytes; HEL, human megakaryocyte-like cell line; macrophages, rat Kupffer cells; platelets, human peripheral blood-derived platelets; HL 60, U937, human promyelocytic leukaemia cell line; MEL, mouse erythroleukaemia cell line; GH_4C_1 pituitary, rat pituitary gland cells; neuroblastoma, mouse neuroblastoma cell line neuro 2a; PC12, pheochromocytoma cells. Abbreviation: n.d., not determined.

Cell line	α	βI/βII	γ	δ	ε	ζ	η	Reference
NIH 3T3	Yes	No	No	Yes?	Yes	Yes	No	H.Mischak (personal communication); T. Sarre (unpublished work)
Rat6 fibroblasts	Yes	No	No	Yes	Yes	Yes	n.d.	Borner et al., 1992
Renal mesangial cells	Yes	No	No	Yes	Yes	Yes	n.d.	Huwiler et al., 1991, 1992
HeLa	Yes	Yes‡	No	No?	Yes	n.d.	n.d.	Pfeffer et al., 1990
preB cells	Yes	Yes‡	No	Yes	Yes?	Yes	Yes?	Mischak et al., 1991a,b
B cells	Yes	Yes‡	No	Yes	Yes?	Yes	Yes	Mischak et al., 1991a,b
T cells	Yes	Yes*	No	Yes	Yes	Yes	Yes	Lucas et al., 1990; Mischak et al., 1991a,b
Plasmacytoma	Yes	Yes?	No	Yes	Yes?	Yes	Yes	Mischak et al., 1991a, b
Myeloid cells	No	Yes?	No	Yes	No	Yes	Yes	Mischak et al., 1991b
HEL	No	Yes‡	No	Yes	No	n.d.	n.d.	Grabarek et al., 1992
Macrophages	No	Yes‡	No	Yes	No	Yes?	n.d.	Duyster et al., 1992; J. Duyster (personal communication)
Platelets	Yes	Yes‡	No	Yes	No	Yes	n.d.	Grabarek et al., 1992; Cook et al., 1992; Crabos et al., 1992
HL 60	Yes	Yes†	No	Yes?	No	Yes	Yes?	Wada et al., 1989; Hashimoto et al., 1990; Hocevar and Fields, 1991; Ways et al., 1992
U937	Yes	Yes‡	No	n.d.	Yes	Yes	n.d.	Wada et al., 1989; Ways et al., 1992
MEL	Yes	No	No	No?	Yes	Yes	n.d.	Powell et al., 1992; T. Sarre (unpublished work)
GH_4C_1 pituitary	Yes	Yes†	n.d.	n.d.	Yes	n.d.	n.d.	Kiley et al., 1990; Akita et al., 1990
Neuroblastoma	Yes	No	No	Yes	Yes	Yes	n.d.	Wada et al., 1989; Bernards, 1991
PC12	Yes	Yes†	Yes	Yes	Yes	Yes	n.d.	Wooten et al., 1992

*β; †βII; ‡βI or βII not determined.

(reviewed by Nishizuka, 1988; see also Yoshida et al., 1988; Wada et al., 1989). This again indicates that a certain set of PKC isoenzymes is necessary to guarantee the ordered sequence of proliferation and differentiation events which leads to and maintains the characteristics of a given tissue.

INTRACELLULAR DISTRIBUTION AND TRANSLOCATION OF PKC ISOENZYMES

More recently, the distribution of PKC isoenzymes within resting (unstimulated) cells of various origins has been determined. Upon treatment of these cells with certain stimuli, either specific ones (e.g. growth factors, hormones or cytokines) or unspecific ones (e.g. serum, phorbol esters), a redistribution of PKC isoenzymes can be observed. With prolonged treatment, the proteolytic degradation and downregulation (see above) could be investigated, too. The distribution, translocation and downregulation of PKC could be visualized by analysis of the so-called particulate (i.e. membrane) and soluble (i.e. cytosolic) fractions of the cells, by means of PKC activity measurement or immunoblotting (using isoenzyme-specific anti-PKC antibodies).

Following the activation model outlined above, translocation to (the) cellular membrane(s) has been regarded as an equivalent of the activation of the respective PKC isoenzyme. However, in various cell types, rather significant portions of certain PKC isoenzymes are constitutively present in the particulate fraction, and it is hard to believe that this indicates a permanent and persistent activation or activity as has been proposed (Bazzi and Nelsestuen, 1988; Burgoyne, 1989). With cPKC isoenzymes, an increase in intracellular Ca^{2+} concentrations is thought to promote translocation to the cellular membrane where subsequent activation by DAG/PtdSer occurs. Following this line of thought, phorbol esters should not only be persistent activators of PKC within the cellular membrane, but should also exert a pleiotropic effect that leads to translocation of the respective isoenzymes. Moreover, phorbol ester treatment leads to the translocation of the Ca^{2+}-independent nPKC isoenzymes, too, a mechanism which is not understood at all. Recent observations that phosphoinositides as such are capable of activating PKC *in vitro* (Chauhan et al., 1991; Lee and Bell, 1991; Kochs et al., 1993) allow for the possibility that translocation alone, without the additional production of second messengers and activators, may lead to the activation of the respective PKC isoenzyme.

In most cell lines investigated, e.g. those depicted in Table 3, PKC α seems to be located in the cytosol and is translocated to the cellular membrane and downregulated upon PMA treatment (Hocevar and Fields, 1991; Strulovici et al., 1991; Huwiler et al., 1991; Crabos et al., 1991; Borner et al., 1992). With more specific stimuli, however, remarkable differences are observed. In GH_4C_1 pituitary gland cells, PKC α is translocated to the particulate fraction, but not downregulated upon treatment with thyrotropin-releasing hormone (TRH) or PMA (Akita et al., 1990; Kiley et al., 1990). Likewise, the PKC α content of neuronal PC12 cells is unchanged over a 10 day period in the presence of nerve growth factor (NGF) (Wooten et al., 1992). In NIH 3T3 cells, PKC α is reported to translocate to the nucleus upon PMA treatment (Leach et al., 1989), and in Swiss 3T3 cells, PKC (presumably PKC α as the major isoenzyme in fibroblasts) seems to associate with the nuclear fraction upon treatment with insulin-like growth factor I, but with the cellular membrane in the presence of bombesin (Divecha et al., 1991). As reported recently (James and Olson, 1992), a PKC α mutant lacking the regulatory domain was associated primarily with the nuclear envelope.

PKC β also appears to be a cytosolic isoenzyme in unstimulated cells which is sensitive to downregulation by PMA (Huang et al., 1989; Akita et al., 1990; Kiley et al., 1990; Strulovici et al., 1991; Crabos et al., 1991; Cook et al., 1992; Duyster et al., 1992). It should be noted, however, that statements like that should not be generalized since, recently, Van den Berghe et al. (1992) reported the PMA-resistant localization of PKC βI in a human colonic cell line. In GH_4C_1 pituitary gland cells, PKC βII is down-

regulated by PMA, but not by TRH treatment (Akita et al., 1990; Kiley et al., 1990). Stimulation of human platelets with IL-3 leads to a rapid translocation of PKC β that is more pronounced than that observed with PKC α (Cook et al., 1992), and IFN-α induces a selective translocation of PKC β to the particulate fraction in HeLa cells (Pfeffer et al., 1990). In HL 60 cells treated with retinoic acid (Makowske et al., 1988; Hashimoto et al., 1990) and in PC12 cells under long term treatment with NGF (Wooten et al., 1992), PKC βII is significantly accumulated whilst the other isoenzymes are downregulated or unchanged. Recently, Tanaka et al. (1992) reported an increase in the activities of PKC α and β during the differentiation of HL 60 cells and the appearance of a 'new' PKC isoenzyme when cells were induced to differentiation by retinoic acid. In rat liver and in HL 60 cells, a distinct portion of PKC β is reported to be found in the nuclear fraction (Klsa et al., 1988; Rogue et al., 1990; Hocevar and Fields, 1991).

Depending on the cell type, PKC δ seems to be differentially distributed within the cell. Whilst located in the cytosol in human platelets (Grabarek et al., 1992), the majority of PKC δ is found associated with the particulate fraction in rat6 fibroblasts (Borner et al., 1992) and in renal mesangial cells (Huwiler et al., 1992). In the latter cell lines, the isoenzyme can be completely downregulated by PMA treatment (Borner et al., 1992; Huwiler et al., 1992).

Rather controversial observations have been made with PKC ϵ, which is mostly cytosolic in GH_4C_1 pituitary gland cells (Akita et al., 1990; Kiley et al., 1990) and human neuroblastoma cells (A. Javala and K. Akerman, personal communication), but membrane-associated to a certain extent in U937 cells (Ways et al., 1992b), rat6 fibroblasts (Borner et al., 1992) and renal mesangial cells (Huwiler et al., 1991). In the latter cell lines, downregulation by PMA is significantly diminished (Huwiler et al., 1991; Borner et al., 1992), whilst in the promonocytic cell line U937 as well as in thymocytes, PKC ϵ is resistant to downregulation by prolonged PMA treatment (Strulovici et al., 1991). In contrast to PKC α and βII, PKC ϵ is downregulated in GH_4C_1 pituitary gland cells by both PMA and TRH treatment (Akita et al., 1990; Kiley et al., 1990). IFN-α treatment of Daudi cells has been reported to result in a selective and rapid activation of PKC ϵ (Pfeffer et al., 1991).

In several cell lines investigated so far, PKC ζ seems to be present as a cytosolic isoenzyme (Crabos et al., 1991; Borner et al., 1992; Huwiler et al., 1992); only in HL 60 cells, has it been reported to be mostly in the particulate fraction (Ways et al., 1992a). Again, translocation and downregulation by PMA appears to be dependent on the cell type. Whilst it is sensitive to PMA treatment in rat6 fibroblasts (Borner et al., 1992) and human platelets (Crabos et al., 1991), it is resistant in HL 60 (Ways et al., 1992a) and renal mesangial cells (Huwiler et al., 1992) and in two neuronal cell lines (Wada et al., 1989; Wooten et al., 1992). Sensitivity towards PMA treatment seems quite contradictory to the fact that PKC ζ neither binds to nor can be activated by phorbol esters (Ono et al., 1989a; Liyanage et al., 1992; McGlynn et al., 1992). However, especially for the nPKC isoenzymes, mechanisms of activation and degradation must be postulated which might involve other cellular (maybe cell type-specific) components; in fact, membrane-associated PKC-binding proteins have been reported which may serve to anchor or compartmentalize PKC isoenzymes to different intracellular membranes (Wolf and Baggiolini, 1990; Mochly-Rosen et al., 1991).

In several human tumour cell lines, PKC η has been reported to be present specifically in the cell nucleus; PMA treatment does not lead to downregulation (Oreif et al., 1992). When PKC θ was expressed in COS cells, the majority of the protein was found in the particulate fraction (Osada et al., 1992).

Concerning the data accumulated above, we have to admit to being far away from a clear-cut picture of the distribution, translocation and degradation of PKC isoenzymes within distinct signalling pathways in different cells. Likewise, the concept of a "cell depleted of PKC by prolonged PMA treatment" has to be re-evaluated in the light of significant differences in PMA sensitivity between the PKC isoenzymes in various cells. However, a connection of the above findings with data on PKC-mediated proliferation and differentiation events may bring us closer to detailed insights.

In order to investigate the role of distinct PKC isoenzymes as such and, especially, in the control of cell proliferation, PKC isoenzyme cDNAs have been stably overexpressed in several mammalian cell systems under the control of various promoters. In general, the effects observed depend very much on the cell lines and the PKC isoenzyme cDNAs used.

Bovine PKC α has been overexpressed in Swiss 3T3 cells (Eldar et al., 1990) and murine PKC α in BALB/c-, rat6- and ψ2-fibroblasts (Borner et al., 1991). In all cases, no transformation could be observed by growing the cells in soft agar. Overexpression of human PKC α in NIH 3T3 fibroblasts resulted in a slightly transformed phenotype (Finkenzeller et al., 1992) which might be due to the parental cell line used.

Overexpression of rat PKC β1 in rat6 fibroblasts gave rise to transformation, especially in the presence of PMA (Housey et al., 1988), and phospholipase D activity and DAG formation were increased (Pal et al., 1991). In contrast, overexpression of PKC β1 in rat liver epithelial cells was not sufficient by itself for cell transformation (Hsieh et al., 1989). No alterations were observed by overexpressing rat PKC βI in C3H 10T1/2 cells (Krauss et al., 1989), whereas PMA exerted even an inhibitory effect on cell proliferation in HT29 colon cancer cells overexpressing the same PKC isoenzyme (Choi et al., 1990).

Rat PKC γ overexpression in NIH 3T3 fibroblasts may (Persons et al., 1988) or may not (Cuadrado et al., 1990) result in transformation. This may be due to different expression levels of rat PKC γ in these recombinant cell lines or to variations in the parental NIH 3T3 clone used and shows the general difficulty in interpreting effects of overexpressed proteins. When rat PKC γ was transiently cotransfected with a test gene under the control of the VL30 enhancer element it was shown that PKC γ could transactivate the murine VL30 enhancer element (Persons et al., 1991).

Overexpression of the murine PKC δ in NIH 3T3 cells gave rise to a slower cell growth rate, whereas overexpression of murine PKC ϵ in the same cell line led to an increased growth rate (H. Mischak, personal communication). Data on the overexpression of other nPKCs are not available at present.

Usually, overexpression of PKC isoenzymes in mammalian cells is correlated with an enhanced expression of early proto-oncogenes such as *c-jun*, *c-fos*, *c-myc* etc. Very often cells display an altered morphology such as an increase in refractility. In rat6 fibroblasts, it was possible to overexpress PKC β1 (Housey et al., 1988) and α (Borner et al., 1991) 40–50-fold at the protein level, whereas the maximal expression level in other cell lines was up to 10-fold, so far. The PKC βI-overexpressing rat6 cell line (Housey et al., 1988) is the only PKC-overexpressing cell line that is known to give rise to tumours in nude mice. In general, it can be concluded that overexpression of PKC can lead to altered cell growth, but the type of alterations are dependent on other cellular factors.

Rather elegant experiments have been carried out by Ohno et al. (1991): rat fibroblasts were cotransfected with different PKC

cDNAs and the cDNA for the bacterial chloramphenicol acetyltransferase (CAT), the latter under the control either of a TPA-response element (TRE) or a serum-response element (SRE). PKC α, βII and ϵ were able to enhance significantly the expression of both the TRE/CAT and the SRE/CAT genes as well as of the endogenous *c-fos* gene. In contrast, PKC γ only enhanced the expression of the SRE/CAT gene. These findings represent a convincing demonstration of distinct roles of PKC isoenzymes at the level of the control of gene expression in a defined cellular environment.

MODULATION OF GENE EXPRESSION BY PKC ISOENZYMES

Besides analyses on the role of distinct PKC isoenzymes overexpressed in cells stably or transiently transfected with the respective cDNA (see above), several cellular systems have served to elucidate the role of endogenous PKC isoenzymes in the modulation of gene expression during differentiation and proliferation events *in situ*.

T cells

Activation of T cells by antigens, anti-receptor antibodies, IL-1, lectins, PMA, or a combination of these, leads to the expression of IL-2 and the 55 kDa α-subunit of the IL-2 receptor (IL-2Rα) which results in an autocrine stimulation of proliferation (reviewed by Berry and Nishizuka, 1990). In this classical model system, PKC is undoubtedly involved in the activation process, and although a rapid translocation of PKC has been observed, it is clear that PKC activation is required for a prolonged length of time (Berry et al., 1990). In this context, the observation that PKC β, but not PKC ϵ, is downregulated during thymocyte activation by PMA (Strulovici et al., 1991) might gain some significance.

During T cell activation, the expression of the *c-fos*, but not the *c-jun*, gene product is enhanced; these two proteins, as homo- or heterodimeric complexes, represent the transcription factor AP1 which confers inducibility by PMA to genes containing a TRE (TPA-response element) within their promoter DNA sequence (for details see Berry and Nishizuka, 1990; Karin, 1992). Activation of the DNA-binding activity of AP1 seems to involve both PKC-mediated phosphorylation of c-Fos and dephosphorylation of c-Jun (reviewed by Karin 1992; Meek and Street 1992); thus, activated PKC, via the modulation of AP1 activity, may enhance transcription of the IL-2 and IL-2Rα genes. Both genes also exhibit binding sites for the transcription factor NF-κB which, in its inactive cytosolic form, is complexed with an inhibitor of its DNA-binding activity named I-κB; the latter has been reported to be removed from the inactive complex by a phosphorylation event, most probably mediated by PKA and/or PKC by an indirect mechanism (Shirakawa and Mizel, 1989; Grab and Baltimore, 1990; Link et al., 1992). Thus, PKC could be involved in transcriptional activation of the IL-2 and IL-2Rα genes in a dual way, via AP1 and NF-κB, maybe mediated by two different PKC isoenzymes (see below).

Recent reports have pointed out the possibility that two further components of the signal transduction pathway (see Figure 1) are involved in PKC-mediated T cell activation. A significant increase of the G protein p21^{ras} in its GTP-binding (active) form has been observed, which in turn indicates a diminished activity of the so-called GTPase activating protein GAP, due to (direct or indirect) phosphorylation of GAP by PKC (Downward et al., 1990). Another cytoplasmic Ser/Thr kinase, c-Raf kinase, resembles PKC in that it represents a group of closely related isoenzymes also structured into a regulatory and catalytic domain, and containing a zinc finger motif (Morrison et al., 1991; Bruder et al., 1992). Its significant role within the signal transduction pathway has been unequivocally established, although details remain obscure (reviewed by Li et al., 1991).

As to T cell activation, Siegel et al. (1990) could demonstrate the concomitant phosphorylation and activation of the c-Raf kinase via a PKC-mediated pathway; this is supported by recent evidence that c-Raf kinase is a direct substrate of PKC *in situ* and *in vitro* (W.Kolch and G.Kochs, personal communication; Sözeri et al., 1992). Since expression of AP1-driven promotors and genes (see above) is thought to require c-Raf kinase (Bruder et al., 1992), this might indicate, at least for the case of T cell activation, the existence of a protein kinase cascade consisting of one (or more) PKC isoenzyme(s) and the Raf-1 kinase.

Erythroleukaemia cells

Mouse erythroleukaemia (MEL) cells represent a transformed cell line from the erythroid lineage of the haematopoietic system, most probably from the proerythroblast stage. MEL cells can be induced *in situ* to terminal erythroid differentiation marked by the cessation of proliferation, the onset of globin mRNA and haemoglobin synthesis and the expression of several other erythroblast marker genes (reviewed by Reuben et al., 1980). Recently, Melloni et al. (1989) convincingly demonstrated that PKC is involved in, if not responsible for, the onset of differentiation (so-called commitment). Moreover, in this system, the (transient?) presence of a proteolytically activated, cytosolic form of PKC (see above) seems to be important, as has been demonstrated by the inhibition of differentiation in the presence of the protease inhibitor leupeptin (Melloni et al., 1987). Since levels of PKC α remained unaltered, but PKC βI/βII decreased concomitantly with the onset of differentiation, the latter isoenzyme(s) seemed to exert the key role (Melloni et al., 1989). Re-evaluation of the PKC isoenzyme pattern of MEL cells, however, has revealed the absence of any PKC β isoenzyme, but a significant abundance of PKC ε (Powell et al., 1992; T. Sarre, unpublished work), which may now be the candidate isoenzyme during commitment. A potential role of PKC ε in T cell activation (see above) may be mere coincidence as well as the fact that cells from the non-erythroid myeloid lineage (see below), which cannot be induced to erythroid differentiation, lack this isoenzyme, but express PKC β which is not present in MEL cells (Table 3). Recently, a rapid elevation of DAG levels, followed by a significant decrease, has been reported as one of the earliest events during MEL cell differentiation (Michaeli et al., 1992).

Rather little is known of the modulation of gene expression linked to erythroid differentiation. In differentiating MEL cells, downregulation of both *c-myc* and *c-myb* gene expression has been reported (D. Eick, personal communication; Smith et al., 1990; Danish et al., 1992) which contrasts with the observation that erythroid differentiation achieved by erythropoietin in aplenic erythroid cells is accompanied by an increase of *c-myc* RNA which seems to depend on the activation of PKC (Patel et al., 1992).

Involvement of PKC in the modulation of gene expression at the translational rather than the transcriptional level has been neglected for some time, though changes in both the proliferative and differentiation status of cells are accompanied by significant alterations of the overall rate and quality of protein biosynthesis (reviewed by Hershey, 1991). For example, differentiation of MEL cells leads to a decrease of the global protein synthesis rate

to about 30%, though in parallel globin synthesis reaches a maximum (Bader and Sarre, 1986). The dominant mechanisms of translational control involve reversible phosphorylation reactions on components like ribosomal proteins, initiation or elongation factors (Hershey, 1991). The ribosomal protein S6 (of the 40 S subunit) as well as the initiation factors eIF-3, eIF-4E, eIF-4B and eIF-4F (the mRNA cap binding complex) have been reported to be phosphorylated in cells treated with insulin or phorbol esters (Morley and Traugh, 1990). Whilst S6 phosphorylation is exerted by a S6 kinase which seems to be downstream of PKC in a protein kinase cascade (Susa et al., 1989, and references cited therein), at least eIF-4F and -4E appear to be direct substrates of PKC *in situ* and *in vitro* (Morley et al., 1991; Smith et al., 1991). Interestingly, overexpression of the cDNA encoding eIF-4E in NIH 3T3 cells lead to cellular transformation (Lazaris-Karatzas et al., 1990) and expression of antisense cDNA diminished the proliferation rate of HeLa cells (De Benedetti et al., 1991).

Thus, it seems likely that translational control mechanisms involved in proliferation and differentiation events are exerted in part, directly or indirectly, by activated PKC isoenzymes.

Myeloid cells

A third *in situ* system used for the analysis of the role of PKC in the control of differentiation involves human myeloid or promyelocytic leukaemia cell lines (HL 60, U937, K562, HEL; see Table 3). These cells tend to differentiate into distinct cell types depending on the inducer used: upon exposure to phorbol esters, HL 60, K562 and U937 cells show a series of monocyte/macrophage properties, whilst HL 60 cells treated with retinoic acid or DMSO exhibit a neutrophil-like (granulocyte) phenotype (Hass et al., 1991; Okuda et al., 1991; Ways et al., 1992a,b; and references cited in Table 3). In contrast, K562 cells differentiate along the erythroid lineage upon treatment with haemin (Rutherford et al., 1979; Okuda et al., 1991). Following PMA treatment, the megakaryocyte-like HEL cells develop properties characteristic of platelets and megakaryocytes (Grabarek et al., 1992, and references cited therein). As is the case for T cells (Berry et al., 1990), HL 60 cells require a sustained activation of PKC for differentiation to the macrophage phenotype (Aihara et al., 1991). This is in agreement with the observation that high concentrations of PMA lead to differentiation of HL 60 cells whilst low doses of phorbol ester are just mitogenic (Trayner and Clemens, 1992), a finding which might reflect distinct roles in proliferation and differentiation events of PKC isoenzymes differing in their susceptibility towards PMA. The tremendous redistributions and changes in abundance of PKC isoenzymes observed during the differentiation processes in these cells (see above) indicate that PKC isoenzymes seem to play an important role, which will be worthwhile to elucidate.

PERSPECTIVES

An overwhelming amount of information on PKC has accumulated since its discovery in 1977. As outlined in this Review, successful work has been carried out with respect to the number of isoenzymes, their structure and biochemical properties, their expression, localization and activation within resting, proliferating and differentiating cells. In order to understand the divergent role of PKC isoenzymes in distinct signal transduction pathways, however, several aspects will require special attention in the future and intensive investigations both *in vitro* and by the use of appropriate cellular systems.

Physiological inducers of a PKC response should get preference to artefactual inducers potentially exerting pleiotropic (or no) effects. Thus, in the light of the extremely different sensitivity of PKC isoenzymes to long-term PMA treatment *in situ* (see above), the experimental paradigm of 'PKC downregulation' should be handled with care (see also Varese et al., 1992). Instead, a detailed analysis of the activation of distinct PKC isoenzymes in cells which respond to a specific inducer with a distinct differentiation program will provide us with much more profound insights. Recently, Szamel and Resch (1992) demonstrated that early in T cell stimulation (see above), PKC α is transiently activated whilst a prolonged activation of PKC β is observed after a lag phase. PKC α activation appeared to be sufficient for the expression of IL-R_α, whereas the sustained activation of PKC β was a prerequisite for the expression of IL-2.

Although there are potent PKC inhibitors available now (reviewed by Tamaoki and Nakano, 1990), the development of isoenzyme-specific PKC inhibitors will be extremely useful. As described above, stable overexpression of PKC δ in NIH 3T3 cells leads to a diminished growth rate. Interestingly, in these cells normal proliferative behaviour could be restored by an inhibitor for both cPKC and nPKC, but not by a cPKC-specific inhibitor (G. Martiny-Baron and C. Schächtele, personal communication). Likewise, the inhibition of the synthesis of individual PKC isoenzymes by the use of specific oligoribonucleotides as well as the intracellular use of isoenzyme-specific antibodies will allow for the depletion and inactivation respectively of distinct PKC isoenzymes. Recently, Leli et al. (1992) demonstrated that intracellular delivery of anti-PKC α and PKC ε antibodies into transiently permeabilized neuroblastoma cells was sufficient to induce differentiation.

With regard to the group of nPKCs, the biochemical nature of second messengers generated by different incoming signals has to be determined. Correspondingly, the activator and cofactor requirements of the individual PKC isoenzymes both *in vitro* and *in situ* have to be elucidated. Northern and Western blot techniques with PKC isoenzyme-specific probes will permit the determination of the expression pattern, the localization and redistribution of PKC isoenzymes in different tissues and cells and thus complete our understanding of the complex PKC isoenzyme network.

Transient and stable (over)expression of PKC isoenzyme cDNAs under the control of constitutive and inducible promotors will surely enlarge our understanding, especially if more sophisticated approaches are chosen: (i) expression of a PKC isoenzyme not present in the respective cell type, (ii) co-expression of individual PKC isoenzymes with other (downstream or upstream) components of signal transduction pathways , (iii) expression of PKC isoenzyme mutants, e.g. chimerae of the regulatory and catalytic domains of different isoenzymes or mutants carrying deletions within the V1, the zinc finger and the hinge region, respectively. Promising work with respect to this goal has been initiated, e.g. the cotransfection experiments by Ohno et al. (1991), the PKC ϵ/γ chimera of Pears et al. (1991) and the PKC α devoid of the regulatory domain (James and Olson, 1992). Thus, the key role of PKC isoenzymes in signal transduction and its impact on the modulation of gene expression linked to proliferative and differentiation events will be further substantiated.

We acknowledge the work of numerous colleagues whose contributions to the field may not be mentioned in this Review, due to limits in space and to the scope of this article. We would like to thank Georg Kochs, Walter Kolch, Peter Parker and Marius Ueffing for helpful discussions, suggestions and criticism during the preparation of the manuscript. We are grateful to Jussus Duyster, Dirk Eick, Georg Kochs, Walter Kolch, Georg Martiny-Baron and Harald Mischak for making data available to us prior

to publication. The work in our laboratories is supported by the Bundesministerium für Forschung und Technologie of Germany.

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One page.

1: PRKCB1 — Order cDNA clone, Links

Official Symbol PRKCB1 and **Name:** protein kinase C, beta 1 [*Homo sapiens*]
Other Aliases: MGC41878, PKC-beta, PKCB, PRKCB, PRKCII2
Other Designations: protein kinase C, beta; protein kinase C, beta 1 polypeptide
Chromosome: 16; **Location:** 16p11.2
Annotation: Chromosome 16, NC_000016.8 (23754823..24139063)
MIM: 176970
GeneID: 5579

2: PRKCB1 — Order cDNA clone, Links

protein kinase C, beta 1 [*Bos taurus*]
Other Designations: protein kinase C, beta; protein kinase C, beta 1 polypeptide
Chromosome: 26
Annotation: Chromosome 25, NC_007326.2 (22886068..23256517)
GeneID: 282325

3: Prkcb1 — Order cDNA clone, Links

Official Symbol Prkcb1 and **Name:** protein kinase C, beta 1 [*Mus musculus*]
Other Aliases: A130082F03Rik, PKC-Beta, Pkcb, Prkcb, Prkcb2
Other Designations: protein kinase C beta-II
Chromosome: 7; **Location:** 7 60.0 cM
Annotation: Chromosome 7, NC_000073.5 (129432639..129777916)
GeneID: 18751

4: prkcb1 — Order cDNA clone, Links

Official Symbol prkcb1 and **Name:** protein kinase C, beta 1 [*Xenopus tropicalis*]
Other Aliases: PKCbeta, prkcb
GeneID: 100038043

5: Prkcb1 — Links

Official Symbol Prkcb1 and **Name:** protein kinase C, beta 1 [*Rattus norvegicus*]
Other Aliases: Pkcb
Other Designations: protein kinase C beta I; protein kinase C beta II; protein kinase C, beta
Chromosome: 1; **Location:** 1q36
Annotation: Chromosome 1, NC_005100.2 (181117515..181459856)
GeneID: 25123

6: PRKCB1 — Links

protein kinase C, beta 1 [*Oryctolagus cuniculus*]
GeneID: 100038311

7: PRKCB1 — Links

protein kinase C, beta 1 [*Sus scrofa*]
Other Designations: protein kinase C beta 2
GeneID: 397183

8: PRKCB1 — Links

protein kinase C, beta 1 [*Oryctolagus cuniculus*]
GeneID: 100037719

9: prkcb1 — Order cDNA clone, Links

Official Symbol prkcb1 and **Name:** protein kinase C, beta 1 [*Danio rerio*]
Other Aliases: MGC63591, Prkcb, zgc:63591
Chromosome: 3
Annotation: Chromosome 3, NC_007114.2 (38072834..38784594, complement)
GeneID: 393953

10: PRKCB1 — Links

protein kinase C, beta 1 [*Gallus gallus*]
Other Designations: protein kinase C beta 1
Chromosome: 14
Annotation: Chromosome 14, NC_006101.2 (6715547..6772687)
GeneID: 416567

11: PRKCB1 — Links

protein kinase C, beta [*Macaca mulatta*]
Chromosome: 20
Annotation: Chromosome 20, NC_007877.1 (22314488..22688170)
GeneID: 701195

12: PRKCB1 — Links

protein kinase C, beta 1 [*Canis lupus familiaris*]
Chromosome: 6
Annotation: Chromosome 6, NC_006588.2 (24761572..24961681, complement)

GeneID: 489968

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